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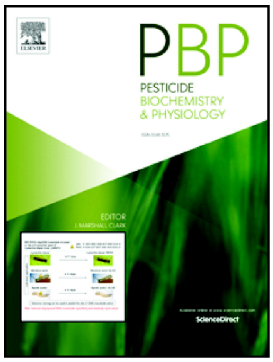

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Target-site mutations (*AChE* and *kdr*), and PSMO activity in codling moth (*Cydia pomonella* (L.) (Lepidoptera: Tortricidae)) populations from Spain

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A B S T R A C T

Codling moth, *Cydia pomonella* (L.) is a key pest of global importance that affects apple fruit production and whose populations have developed resistance to insecticides in many apple production areas. In Spain, enhanced cytochrome P450 polysubstrate monooxygenase (PSMO) activity is the main mechanism involved in insecticide detoxification by codling moth, although acetylcholinesterase (AChE) target site mutations have been described in two populations. However, the extent of *AChE* and knockdown resistance (*kdr*) mutations in Spain is unknown. To assess the actual occurrence of *AChE* and *kdr* mutations concurrently with the frequency of moths with PSMO enhanced activity (R-PSMO), 32 Spanish field populations from four apple-growing areas of Spain and two susceptible laboratory strains were evaluated. R-PSMO was significantly higher in 23 chemically treated field populations from Extremadura, Catalonia and Aragon, with proportions that varied between 25% and 90%, but no significant differences among strains and the non-chemically treated orchards (organic or abandoned) were observed. The *AChE* mutation (F290V) was detected in all field populations from Catalonia (n=21) and in three field populations from Aragon (n=5), with resistant phenotype proportions varying from 34.2% to 97.5% and from 7.2% to 65% in Catalonia and Aragon, respectively. In addition, the *kdr* mutation (L1014F) was detected in twelve Catalonian field populations, at rates of incidence ranging between 2.6% and 56.8%. A positive correlation between R-PSMO and *AChE* mutation was found. The origin of the mutations and their ability to persist and spread in field populations with different management systems is discussed.

Key words: insecticide resistance, *AChE* and *kdr* mutations, frequency of moth with PSMO enhanced activity (R-PSMO), Spanish apple-growing areas, management types.

1. Introduction

A key component of the integrated management of key pests such as codling moth (*Cydia pomonella* (L.), Lepidotera: Tortricidae) is establishing an insecticide-resistance management (IRM) program. Codling moth is one of the most damaging pests of pome fruit crops worldwide, although in almost all the areas where such crops are cultivated it affects mainly apple production [1]. As a result of long-term pesticide use, the codling moth has developed resistance to different insecticide modes of action and chemistries, such as neurotoxic insecticides and insect growth regulators [2-18], and even to *C. pomonella* granulovirus (CpGV) (family Baculoviridae) [19].

Worldwide, codling moth insecticide resistance is mainly associated with the increased activity of detoxifying enzymes such as non-specific esterases (ESTs), cytochrome polysubstrate P450 monooxygenases (PSMOs), and glutathione S-transferases (GSTs) [3,14,20-22]. In addition, two target-site mutations (structural changes in the insecticide target proteins that render them less sensitive to an insecticide) have been reported: a F290V replacement in acetylcholinesterase in *AChE-1* gene, *AChE* onwards, involved in resistance to organophosphates and carbamates [23], and a L1014F replacement in the voltage-gated sodium channel gene, *kdr* onwards, involved in resistance to pyrethroids [24]. The *AChE* mutation confers phenotypic resistance under both homozygote and heterozygote conditions, whereas the *kdr* mutation confers it only under the recessive homozygote condition.

In codling moth Spanish field populations, insecticide resistance has been associated with three detoxification systems, mainly PSMO in adults and larvae [14-15,21,25-26], and to a lesser extent GST and EST in larvae [14-15,25]. As for target-site mutations, Reyes *et al.* [21,27] found the *AChE* mutation only in a single Spanish field population

from a Catalan (NE Spain) apple-growing area, in a study of the variability of resistance mechanisms worldwide that involved a total of 55 populations from Europe (1 from Spain) and 24 from other continents. This Catalan population came from the same area where the Raz population, selected in laboratory with azinphos-methyl by INRA in Avignon, was sampled and in which the *AChE* mutation was identified by Cassanelli *et al.* [23]. The *kdr* mutation was not detected in this Spanish field population [21,27-28]. The existence of multiple resistance mechanisms increases the difficulty of codling moth control and it interferes with the management programs in the orchards. Brent [29] pointed out that the existence of at least 5% of resistant individuals is required in a population to detect a resistance problem, but Denholm *et al.* [30] and Hoy [31] reported that resistance is very difficult to manage even when only 10% of the individuals carry resistance genes. It is therefore important to establish an IRM program for Spanish codling moth populations to avoid or delay the increase in the frequency of resistant individuals. This requires the early detection of the existing resistance mechanisms in field populations and knowledge of their extent at local scale.

Adult moths with PSMO enhanced activity (PSMO-resistant onwards) from Spanish populations did not show a greater attraction to pure kairomone-baited traps in apple orchards [32], as was reported by Sauphanor *et al.* [33] for French PSMO-resistant populations. However, PSMO detection in codling moth adults caught during the different flights, in orchards under different crop management systems, was found to be a good tool to assess levels of insecticide resistance in field apple crops [32]. Extensive studies of increases in the metabolic capacity of detoxifying enzymes have been carried out in the Ebro Valley pome fruit production area [14-15,25], but not on the extension in the occurrence of the target site sensitivity.

In an attempt to complete the overview of resistance mechanisms in Spanish codling moth populations, the aims of the present work were to assess the actual occurrence of *AChE* and *kdr* mutations in codling moth field populations from different pome fruit-growing areas of Spain, concurrently with the frequency of moths with PSMO enhanced activity (R-PSMO).

2. Materials and methods

2.1. Insects

Thirty-two codling moth field populations were collected during the years 2010 to 2012 in four Spanish autonomic regions where pome fruits are cultivated (Figure 1): Asturias (AST), n=5; Extremadura (EXT), n=1; Aragon (ARA), n=5; and Catalonia (CAT) n=21 (Table 1). Apple cultivation in Asturias (4,106 ha in 2016) is dedicated to cider production, and orchards are managed mainly organically and without irrigation. In the other three regions, apple trees are grown for table apple production, and orchards are intensively managed. In 2016, Catalonia (11,066 ha, mostly in Lleida) and Aragon (4,576 ha) represented 75% of the total Spanish acreage dedicated to apple production under irrigation.

Codling moth adults were either caught in monitoring delta traps baited with attractants or as they emerged from larvae caught in cardboard traps. According to the intensity of chemical insecticide treatments, the orchards were grouped as non-chemically treated orchards (UN, n=8, abandoned or organic orchards), chemically treated orchards (CH, n=12), and mating disruption plus chemical control orchards (MD+CH, n=12). In several orchards, the percentage of damaged fruits at harvest was higher than 2% in spite of the control measures applied. Two codling moth-susceptible laboratory strains were used: (i) a Spanish susceptible strain, S_Spain (SSp), collected from an abandoned

apple orchard in Lleida in 1992 and reared since then at the joint IRTA (Institute for Food and Agricultural Research and Technology) and UdL (University of Lleida) laboratory (Lleida, Spain) using a semi-artificial dehydrated apple diet, and (ii) a French susceptible strain, S_France (Sv), provided by INRA (Avignon, France), mass-reared on an artificial diet (Manduca Premix-Heliothis Premix, Stonefly Inc., Bryan, TX) under laboratory conditions. SSp was used as the main reference in this study.

2.2. PSMO activity

To determine PSMO activity, freshly emerged adult abdomens were dissected to be used as enzymatic source, as described by Rodríguez *et al.* [26], in 6 g L^{-1} sodium chloride, and were placed in black 96-well microplates. The activity was measured using 7-ethoxycoumarin O-deethylation activity (ECOD), adapted for *in vivo* analysis [34]. The methodology used was the same as described by Reyes *et al.* [21]. The reaction was initiated when an adult abdomen was individually introduced into a well containing 100 mL of phosphate buffer (50 mM, pH 7.2) and ethoxycoumarin (0.4 mM). After 4 h of incubation at 30 ºC, the reaction was stopped by adding 100 mL of 0.1 mM glycine buffer (pH 10.4)/ethanol (v/v). The 7-hydroxycoumarin fluorescence was quantified using a multilabel plate counter VICTOR3 (Perkin Elmer Life and Analytical Sciences, Madrid, Spain), with 380-nm excitation and 450-nm emission filters. In each plate, twelve wells were used as controls and received the glycine buffer prior the incubation. A standard curve was obtained using 7-hydroxycoumarin, and PSMO activity was expressed as pg of 7-hydroxycoumarine $insect^{-1}$ min^{-1}.

2.3. AChE *and* kdr *mutations*

Target-site mutations related to insecticide resistance, located in *AChE-1* and *kdr* genes, were analysed using PCR–RFLP [21,24] in 1,174 adults (1,103 from field populations and 71 from the two susceptible laboratory strains). Total DNA was obtained from the adult thorax, using the "salting out" methodology described by Fuentes-Contreras *et al.* [35]. PCR amplifications of *kdr* and *AChE-1* genes were carried out separately, in a 25-µL reaction volume containing primer reaction buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 200 µM of each dNTP, 0.4 mM of each primer, 1 unit of Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) and 2 µL of DNA template. Restrictions were performed by pooling the PCR products from both genes (5 µL each) with 0.2 units of Tsp509I (New England Biolab, Ipswich, MA, USA) in a 20-µL reaction volume incubated at 65 ºC for 16 h. DNA fragments were separated by electrophoresis on 6% polyacrylamide gel and visualized after silver staining. DNA fragments of 141 bp and 102 bp, respectively, identified the mutant and sensitive *AChE* alleles. The *kdr* mutant allele was identified by a DNA fragment of 77 bp and two different sensitive alleles were identified by fragments of 101 bp and 112 bp, respectively. The genotype frequency of *AChE* and *kdr* mutations was measured.

2.4. Data analysis

The distribution of the variable PSMO activity did not fulfil the assumption of homoscedasticity, and several standard transformations did not normalize it (Normal Q-Q plot and Shapiro-Wilkes normality test), using the *qqplot* and *shapiro.test* functions in R language [36]. Thus, a non-parametric Kruskal–Wallis test was performed, followed by a multiple comparison (post hoc) test [37], using the *kruskalmc* function from the *pgirmess* R package [38] to evaluate PSMO activity among populations and

among orchards grouped by their management system. In both cases, only field populations with $n \geq 20$ adults were used.

To calculate the relative frequency of PSMO-resistant codling moth adults (R-PSMO), an adult was classified as resistant if its PSMO activity was higher than the upper value of the 95% confidence limit of the mean PSMO activity of the susceptible strain SSp [21]. A Pearson chi-square (χ^2) test was used to compare the R-PSMO between each population and the susceptible population SSp using *chisq.test* functions [36].

To detect whether the *AChE* and *kdr* genes were under selection pressure, a Fisher's exact test was performed for each population to check the Hardy-Weinberg equilibrium (GENEPOP 4.5 [39]). Finally, Pearson correlation was used to evaluate the relationship between the R-PSMO, the percentage of resistant insects with the *kdr* mutation and the percentage of resistant insects with the *AChE* mutation. The same relationships were subjected to principal component analysis (PCA) using the *rda* function implemented in the *vegan* R package [40].

3. Results

3.1. PSMO activity and R-PSMO

Table 2 shows the PSMO activity in adults of 32 *C. pomonella* Spanish field populations and two susceptible laboratory strains. The mean PSMO activity was significantly different among codling moth populations (p-value < 0.0001, $\chi^2 = 461.71$, d.f. = 33). In general, the chemically sprayed orchards (CH or MD+CH) showed a PSMO activity significantly higher than the susceptible laboratory strains, except in two cases: VIL (CH) from Aragon and SAS (MD+CH) from Catalonia (Figure 2). No significant differences were found among non-chemically treated orchards and the susceptible strains (Figure 2). The mean PSMO activity was also significantly different

among orchard management system populations (p-value < 0.0001, χ^2 = 369.2, d.f. = 3). No significant differences were found among orchards with or without mating disruption, but a significantly higher PSMO activity was observed in orchards subject to insecticide treatment (CH and MD+CH) compared to non-chemically treated orchards and laboratory susceptible strains (Figure 3). Taking into account the origin of the populations, the resistance ratio (R/S), based on the mean PSMO activity, ranged from 0.7 to 1.6 for Asturias; from 3.9 to 15.6 for Aragon; was 10.1 for Extremadura; and ranged from 2.3 to 18.9 for Catalonia (Table 2). Consistent with PSMO activity, the lowest R/S values corresponded to non-chemically treated orchards, while higher R/S values were found in orchards receiving chemical insecticide treatments.

The frequency of PSMO-resistant *C. pomonella* adults, R-PSMO, was calculated using 37.04 pg of 7-hydroxycoumarin insect^{-1} min^{-1} as the threshold. The R-PSMO ranged from 0.0% (Asturias) to 90.0% (Catalonia) (Table 2). Any of the populations from non-chemically treated orchards showed a R-PSMO significantly higher than for the laboratory SSp strain, whereas all the populations from CH or MD+CH, except one (GRI from Aragon), showed a R-PSMO significantly higher than for the laboratory SSp strain (Table 2).

3.2. AChE *and* kdr *mutations*

The *AChE* mutation, which confers resistance to organophosphate and carbamate insecticides, was detected in all populations from Catalonia (n=21), regardless of orchard management system, and in three populations from Aragon (CH orchards) (Table 3). In populations from Asturias and from orchards with a low number of individuals sampled (BAD, from Extremadura; CAS and GRI, from Aragon), the *AChE* mutation was not detected (Table 3). The highest phenotype proportion of the *AChE*

mutation (proportion of homozygotes + heterozygotes individuals) was 97.5%, in Catalonia (ESC, MD+CH), and 65% in Aragon (TAM, CH). For the *AChE* mutation, significant departure from the Hardy-Weinberg equilibrium was detected in 13 out of 21 field populations, all of them from Catalonia (COL, CAM, TVI, PAR, BUT, LLI, CML, TRO, GAT, INT, TOR, ESC, and JUN; Fisher's exact test, $0.0001 < P < 0.015$) and in the field population TAM (CH) from Aragon (Fisher's exact test, $P = 0.023$).

The *kdr* mutation was detected in the recessive homozygosis condition (resistant phenotype, rr, in Table 3) only in 12 populations from Catalonia. The lowest *kdr* proportion was found in TOR (2.6%), and the highest in TOS (56.8%), both orchards subject to MD+CH management systems (Table 3). In nine out of the 12 populations where the *kdr* mutation was detected, departures from the Hardy–Weinberg equilibrium were observed (COL, PAR, LLI, LIN, CML, TOS, PGR, INT, TOR; Fisher's exact test, $0.0008 < P < 0.044$). Although, *kdr* mutations give resistance to pyrethroid insecticides in the recessive homozygosis condition, in 18 field populations sampled the resistance allele was detected in the heterozygosis condition, in proportions that varied from 2.4% in MIR to 39.5% in GAT, MD+CH orchards (Table 3).

3.3. Relationship between R-PSMO and target site mutations

The R-PSMO was positively correlated to the proportion of individuals with the *AChE* phenotypic mutation ($r = 0.639$, $P = 4.5\ E^{-5}$) (Figure 4). Also, we found a positive correlation between the R-PSMO and the proportion of individuals with the *kdr* phenotypic mutation ($r = 0.485$, $P = 0.0037$) and between the mutations *AChE* and *kdr* ($r = 0.508$, $P = 0.0022$) (Figure 4). The PCA (principal component analysis) was undertaken considering the 32 field populations and the two susceptible strains. The PC1 (65%) was mainly explained by *AChE* and R-PSMO, while the PC2 (24%) was

explained by *kdr* (Figure 5). In general, the PCA representation showed a high degree of association between sprayed field populations (CH, MD+CH) and the frequency of R-PSMO and *AChE* mutations, especially among MD+CH orchards, all of them from Catalonia. However, this association was lower for CH orchards from Aragon (PC1); untreated orchards, alongside susceptible strains, showed no association (Figure 5). The *kdr* relationships (PC2), in general only involved Catalan field populations. This representation also shows some Catalan field populations (COL, LLI, LIN and TOS) separated from the rest in the main clusters. The organic orchard COL showed a moderate percentage of *AChE* mutations compared to other non-chemically (UN) treated orchards, but their *kdr* mutation proportions were comparable with chemically (CH and MD+CH) treated orchards. The chemically treated orchards LLI (CH), LIN and TOS (MD+CH) displayed all the resistance mechanisms studied: *kdr* and *AChE* target site mutations and R-PSMO.

4. Discussion

4.1. PSMO activity

The presence and extent of high PSMO activity in codling moth adults and larvae from field populations collected in chemically treated pome fruit orchards from Catalonia is well known [14-15,26,32]. However, in other Spanish regions it has only been reported for one population from Aragon [15]. The range of the mean PSMO activity value obtained in adults of Catalan codling moth field populations collected from 2004 to 2006, ranged between 65.2 and 582.1 pg of 7-hydroxycoumarin insect^{-1} min^{-1} in orchards with chemical treatments, while the only ecological orchard studied showed 8.8 pg of 7-hydroxycoumarin insect^{-1} min^{-1} [25,26]. The R-PSMO was, in almost all the orchards, 100% of the analysed insects. The mean values obtained in the field

populations collected from 2010 to 2012 are in the same range, but the R-PSMO was lower, ranging between 16.7% (GRI) and 90.0% (ESC), and was higher than 80% just in four out of 24 orchards. The high R-PSMO detected in the 2004 to 2006 field populations could be due to the origin of the populations, as all they came from problematic orchards, and to the extensive use of mating disruption technique in the area since 2007. In 2004-2006, the objective was the detection of the presence of resistant populations, while in 2010-2012 the objective was to obtain an overview of the extent of the resistance mechanisms present in the area. The results of the present study confirm that the PSMO mechanism occurs widely in areas of Spain in which codling moth populations are sprayed with insecticides (Catalonia and Aragon), although a more intensive survey in other Spanish pome fruit growing areas, such as Extremadura, should be performed. In contrast, there were no significant differences between R-PSMO in laboratory strains and from UN orchards (Table 2), whether abandoned or organic orchards (mating disruption and carpovirusine treatment strategies). The R-PSMO occurrence was especially low, significantly lower than for the laboratory strain, in populations from Asturias, due to its pome production system. Apples in Asturias are mainly grown for cider production and are generally organically cultivated. This situation has previously been found in Catalonia only in an isolated organic orchard [15]. Interestingly, the R-PSMO in populations from UN orchards in Asturias and Catalonia were different (from 0% to 7.5% in Asturias, and from 17.5% to 19% in Catalonia). A possible explanation of these results may be the presence of a high proportion of resistant moths in the vicinity of the Catalonia UN orchards acting as a resistant moth reservoir, which indicates the importance of knowing the insecticide resistance levels in codling moth field populations at a local scale.

The R-PSMO was not different between CH and MD+CH orchards. As reported in Bosch *et al.* [32], pheromone mating disruption control system, used to reduce intensity of insecticide sprayed in the orchards, does not affect the proportion of PSMO-resistant adults found in the field with respect to CH orchards. Mating disruption used without the support of chemical treatments is not sufficiently effective in most of the Catalan orchards, due to the high population levels. Growers frequently apply the mating disruption technique when they cannot control the pest using insecticides, while the optimum situation should be to use the mating disruption technique at low population levels [41-42]. Although the applications of chemical treatments in these orchards (MD+CH) is less extensive, this seems not to reduce the selection of resistant individuals even when applying a resistance management strategy based on alternating insecticide applications. Boivin *et al.* [43] suggested the use of alternative methods to chemical treatments to reduce the frequency of resistant individuals in the field. Furthermore, the detection of the reduction of the R-PSMO at plot level is less probable in orchards close to chemically treated ones, either under mating disruption or not, because of the dispersal ability of codling moth adults. However, it seems that the R-PSMO over the years in Catalonia has tended to a general decrease with the use of mating disruption, since any population collected in 2010-2012 showed 100% of R-PSMO.

Differences between sexes were not taken into account in our study. The insects from the orchards of the Catalan area, except the one from Girona (SAS), were adults collected by pheromone traps. Although the attractant was Combo lure the percentage of females captured was very low, having more than 19% of females only in four out of 20 field populations (Table 2). The orchards from Aragon, Extremadura and Asturias were collected with corrugated cardboards and, in this case, the percentage of females

was in all the orchards higher than 40%, except in VEV that was 28.1% (Table 2). Reyes *et al.* [43] reported that codling moth females showed higher PSMO activity than males when expressed per insect, probably due to their larger body size; nevertheless, this differences were not generalized in later studies [17]. Furthermore, Rodríguez *et al.* [14], in field adults from different orchards in the same production area, did not find a clear tendency in the enzymatic activity between sexes.

AChE *and* kdr *mutations*

In the present study, both *AChE* and *kdr* target site mutations were detected in Spanish field populations and were not detected in the two laboratory strains tested (Table 3). Occurrence of the *AChE* and *kdr* alleles was observed in samples collected in both chemically treated (CH, MD+CH) and non-chemically treated (UN) orchards. Only one of the 21 orchards from Catalonia (COL) was managed organically. In this case, the selection for the alleles of resistance must occur outside the orchard, in surrounding sprayed commercial orchards, as mentioned before for R-PSMO. The non-chemically treated orchards may be source or refuge of resistant codling moths only when a high proportion of resistant individuals exists in neighbouring areas. Reyes *et al.* [27] obtained similar results for *kdr* mutation in two untreated field populations from France and Argentina.

Before our study, the *AChE* mutation in the codling moth was described by Cassanelli *et al.* [23] in a laboratory azinphos-methyl resistant strain, reared from a population collected in an apple orchard of the Lleida region (Catalonia); and was also described in one Spanish field population from the same area by Reyes *et al.* [21] when they were studying the diversity of resistance mechanisms in different countries worldwide. Voudouris *et al.* [45] and Reyes *et al.* [17] did not find this mutation in field populations

from Greece and Chile, respectively. In our results, the modified *AChE* mutation was detected not only in all field populations from Lleida, as it was in a previous work [46], but also in one field population from the Girona apple-growing area, 180 kilometres away from Lleida and near to the French border (approximately 62 km). We also found it in three field populations from the Aragon apple-growing area, a distance of from 34 km to 221 km away from the nearest Catalan population. Therefore it is likely that the area of Lleida is the source of the *AChE* mutation (5 populations have more than 90% of phenotypically resistant individuals) and that it has spread to the other areas. We found the *AChE* mutation only in two geographically continuous areas (Catalonia and Aragon), but not in the Asturias area which is separated from the others by approximately 700 km.

The *AChE* mutation was already present in Catalonia in the 1990's, as the SSp, collected in the Lleida apple-growing area, showed a low percentage of the mutation [46], that has not been detected in the present study. This laboratory population did not show significant differences in susceptibility to different insecticidal products or different enzymatic activity levels (EST, GST, PSMO) in comparison to two other susceptible laboratory reference populations from France and Italy [25].

On the other hand, our results show the occurrence of the *kdr* mutation in field populations from the Catalan apple-growing area. The voltage-gated sodium channel mutation, *kdr*, is widely distributed in many European populations [21,27], and Franck *et al.* [28] found independent origins for the *kdr* mutation from different codling moth populations worldwide. This could also be the case for Catalan populations, although Franck *et al.* [28] did not find the *kdr* mutations in the Spanish field population they tested. We observed the occurrence of the *kdr* resistant phenotype (recessive homozygote) in 12 field populations from Catalonia, but 18 field populations showed

the resistant allele. In the organic orchard from Catalonia (COL) not treated with pyrethroids, we found the *kdr* mutation in a proportion comparable to the *kdr* proportions found in CH and MD+CH orchards. This could be due to the low fitness cost of the codling moth *kdr* mutation, as suggested by Boivin *et al*. [47] when they explained the high proportion of *kdr* mutations found in French orchards not treated with pyrethroids.

As with Catalonia, Aragon field populations have been subjected to pyrethroid treatments, but in Aragon the *kdr* mutation was not detected. It is possible that metabolic resistance mechanisms, as enhanced by PSMO activity, play a more important role in pyrethroid detoxification in neonate larvae from Aragon field populations, as previously found by Rodríguez *et al*. [25].

Asturias field populations were free of both mutations, but we attribute this result to the management system of the orchards (all under organic production and with regular use of granuloviruses), concurrent with a restricted gene-flow between Asturias and the other Spanish apple-growing areas, due to long geographical distance and low codling moth dispersion capability. The same argument was mooted by Voudouris *et al*. [45] to explain the lack of the *kdr* mutation in Greece in relation to European codling moth field populations.

Nevertheless, in Spanish field populations we believe that dispersion by adult codling moth adults played an important role in the spread of mutations. Fragmentation of the continuous habitats, orchard type, landscape, and tree canopy structure can increase or decrease the adult codling moth mobility at a local scale [48-51]. The codling moth is regarded as a relatively sedentary pest [45,52-53]; thus its dispersal ability is rather limited. Several studies aimed at examining its dispersal behavior using mark–release–recapture and immunomarking methodologies have found that the adults largely

disperse within 60–80 m [52,54-56], although a small proportion (7.4% to 20.0%) are able to fly up to several km [52]. Immigration of susceptible individuals has been suggested as an important mechanism of insecticide resistance mitigation [27,35,57], and the preservation of susceptible individuals in refuges, within productive growing areas, could also be a determinant factor in delaying the increase in the proportion of insecticide-resistant individuals [58-60]. Moreover, differences in body mass and wing morphology between sexes are indicative of different dispersal capabilities [61] and also of different detoxification insecticide capabilities [4,17,57,62]. Therefore, our results suggest that in addition to knowing the resistance status at local scale, the study of the landscape is very relevant, due to its influence on codling moth dispersion. Nonetheless, movement due to human commercial activities should also be considered, especially in continuous geographical areas. The control of the presence of pests in fruits intended for export is strict, sometimes requiring a European Phytosanitary passport, whereas there is no control for the internal market. This difference in control can affect global codling moth dispersion and consequently, the dispersion of mutations. The codling moth has become a serious quarantine pest for apple exports to some countries and regions, for example Taiwan, Western Australia and China, among others [63-64].

An interesting contribution from this present study to the knowledge concerning codling moth resistance mechanisms in Spanish field populations is the association found between the three resistance mechanisms studied. High R-PSMO and a high proportion of *AChE* and *kdr* mutations were observed in the field populations from Catalonia, and even in a single individual. The combination of different resistance mechanisms has been found in some species in laboratory selected strains (*Musca domestica* L. (Diptera. Muscidae) [65], *Drosophila melanogaster* Meigen (Diptera. Drosophilidae) [66], *Aedes*

aegypti (L.) (Diptera. Culicidae) [67], *Plutella xylostella* (L.) (Lepidoptera. Plutellidae) [68]), but in few species in field conditions (*Culex pipiens* L. (Diptera. Culicidae) [69]). The presence and frequency of codling moth mutations found in the field in this present study was surprisingly wide and makes us ponder their ability to persist in natural populations and the cost of their maintenance. Ffrench-Constant *et al.* [70], in a review of *AChE* mutation studies, said that the acetylcholinesterase enzyme was so efficient that perhaps the resistance associated with the mutations has little effect on its function, and therefore it can be readily maintained in natural populations. In addition, in *D. melanogaster* [66] and *A. aegypti* [67], the level of resistance conferred by a single mutation was low, but increased when there were combined mutations. This could be the reason why the low mutation percentage in the codling moth susceptible laboratory strain, SSp, seemed not to produce any resistance effect, because there was no other resistance mechanism activated in SSp. On the contrary, the codling moth field populations had enhanced enzymatic mechanisms combined with the site action mutation. Another possibility to consider is pre-existing polymorphisms in the field populations before the introduction of the insecticides [71]. These polymorphisms could become unbalanced due to the use of insecticides. Departure from the Hardy–Weinberg disequilibrium were detected in our study in 14 populations for *AChE* and in 9 populations for *kdr*. The insecticide selection was probably the reason for this disequilibrium and the presence of the mutations could also have contributed to it [72]. Since the use of azinphos-methyl (2008) and chlorpiryfos-ethyl (2015) were banned in Spain, the use of chlorpiryfos-methyl and pyrethroids has progressively increased, as it has happened in other areas [13], which implies that the selection of resistant individuals will continue. Although as yet undetected, the presence of unidentified mutations other than *AChE* or *kdr*, which may also confer insecticide insensitivity, as

seen in other insect species, cannot be ruled out in future codling moth studies [45,70,73-74].

The factors that can influence the appearance of resistance mechanisms are complex and variable. Several studies have shown that codling moth resistance mechanisms vary according to the geographical origin of codling moth field populations [26-27], even when the resistance is towards a single insecticide. Furthermore, Li *et al.* [75] found different invasion processes between codling moth field populations from northeastern and northwestern China. Even biotic (sex) and abiotic (temperature) factors can influence the fitness–cost of resistance and therefore its evolution [71].

5. **Conclusions**

Codling moth field populations from the Ebro Valley (Catalonia and Aragon), which was the area most sampled, exhibited a wide range of defence responses to insecticide applications, as evidenced by the high frequency of insects with enhanced PSMO and the two identified target-site mutations, *kdr* and *AChE*. In contrast, the field populations from Asturias did not present any of these mechanisms. Ebro Valley is the only area globally with an extensive distribution of the codling moth *AChE* mutation. In the past, other areas of apple production have also received intensive insecticide treatments with organophosphates and carbamates to control the pest but genetic mutations have not been detected, perhaps because they have not been researched sufficiently intensively. The rapid increase in molecular biological information will probably lead to the discovery of as yet unidentified mutations in the most important pest species, the codling moth among them.

Studies on the genetic structure of the codling moth's Spanish field populations are needed to investigate the possibility of different codling moth origins in the different

Spanish regions, so as to explain the presence of the *AChE* mutation in just one area, and also to explore the variability of fitness costs in the field populations, as dependent upon the geographical origin, the management system and the resistance mechanisms activated.

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Figure captions

Figure 1. Sampling sites locations. Dark areas represents four Spanish autonomic regions (Asturias: Asturias province; Extremadura: Badajoz province; Aragón: Huesca and Zaragoza provinces; Catalonia: Lleida and Girona provinces).

Figure 2. Box plot showing PSMO enzyme activities of 29 field populations and two susceptible strains based on management orchards systems (LAB: laboratory strains, UN: non-chemically treated orchards, CH: chemically treated orchards, MD+CH: mating disruption plus chemical treated orchards) using non-parametric Kruskal-Wallis multiple comparison test (AST: Asturias, ARG: Aragón and CAT: Catalonia apple growing areas).

Figure 3. Box plot showing PSMO enzyme activities of 29 field populations and two susceptible strains (LAB) based on management orchards systems (UN: non-chemically treated orchards, CH: chemically treated orchards, MD+CH: mating disruption plus chemical treated orchards) using non-parametric Kruskal-Wallis multiple comparison test.

Figure 4. Pearson correlations between frequency of resistant insects (R-PSMO), frequency of *kdr* mutation and frequency of *AChE* mutation, from 32 Spanish field populations and two laboratory susceptible strains of *Cydia pomonella* (L.) data.

Figure 5. Principal components analysis for the frequency of resistant individuals (R-PSMO), and frequency of *AChE* and *kdr* target site mutations, from 32 field populations and two laboratory susceptible strains of *Cydia pomonella* (L.) data (LAB: laboratory strains, UN: non-chemically treated orchards, CH: chemically treated orchards, MD+CH: mating disruption plus chemical treated orchards).

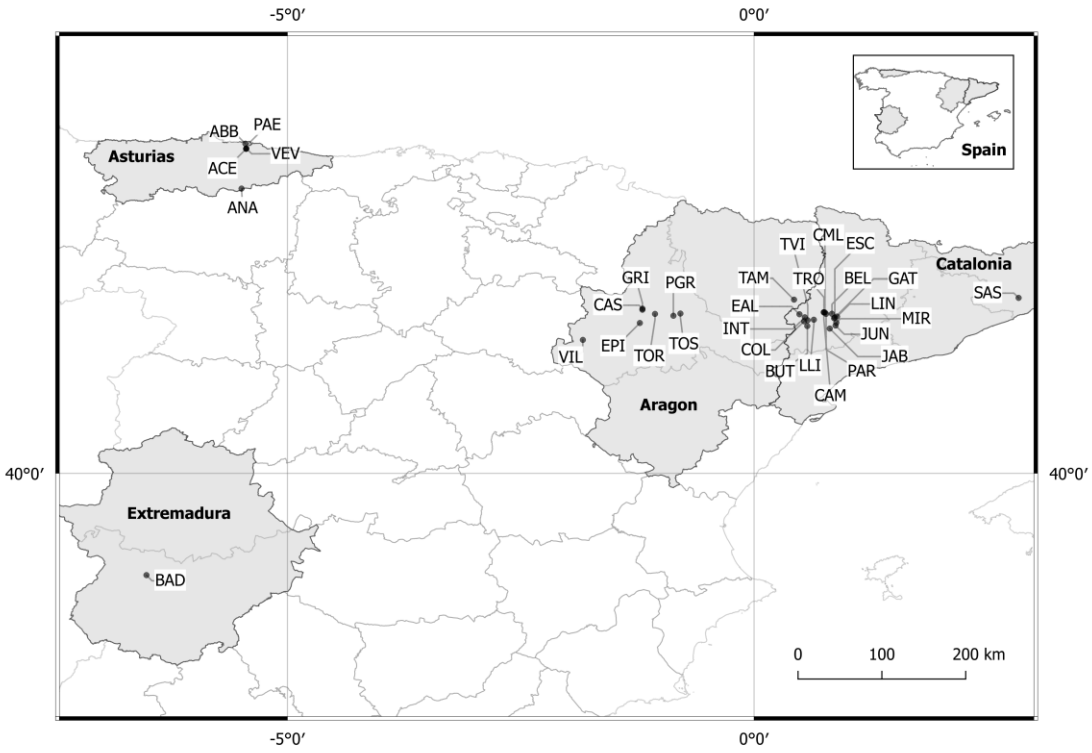


Figure 1

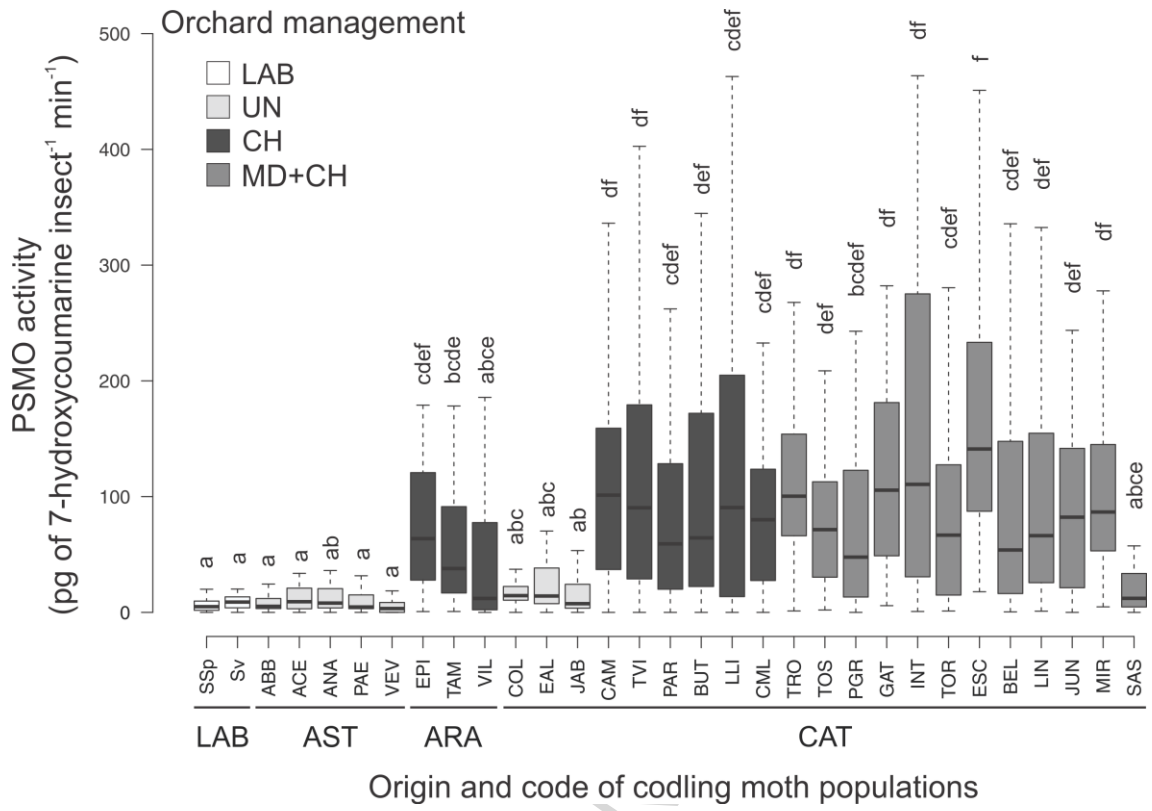


Figure 2

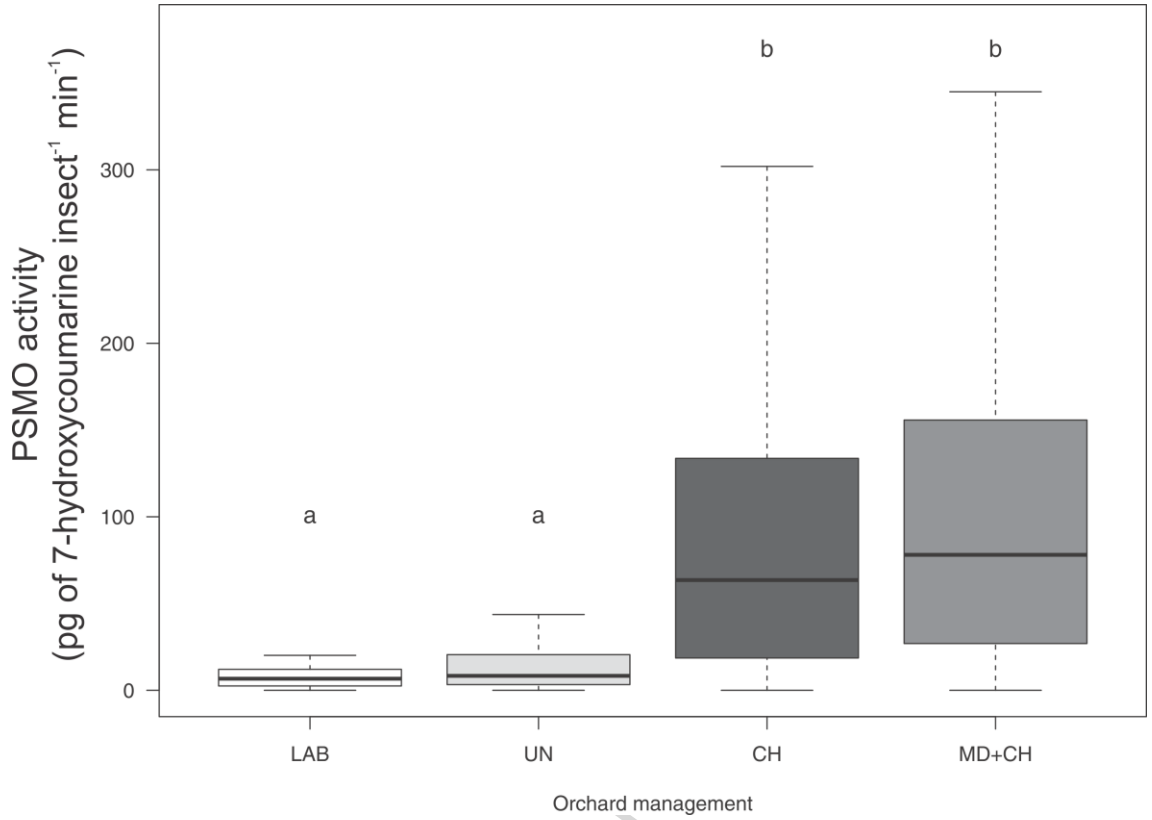


Figure 3

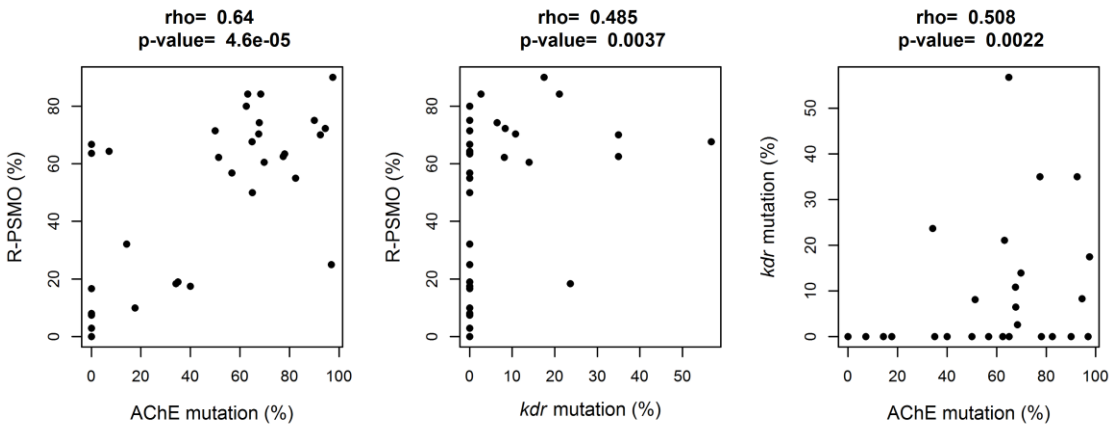


Figure 4

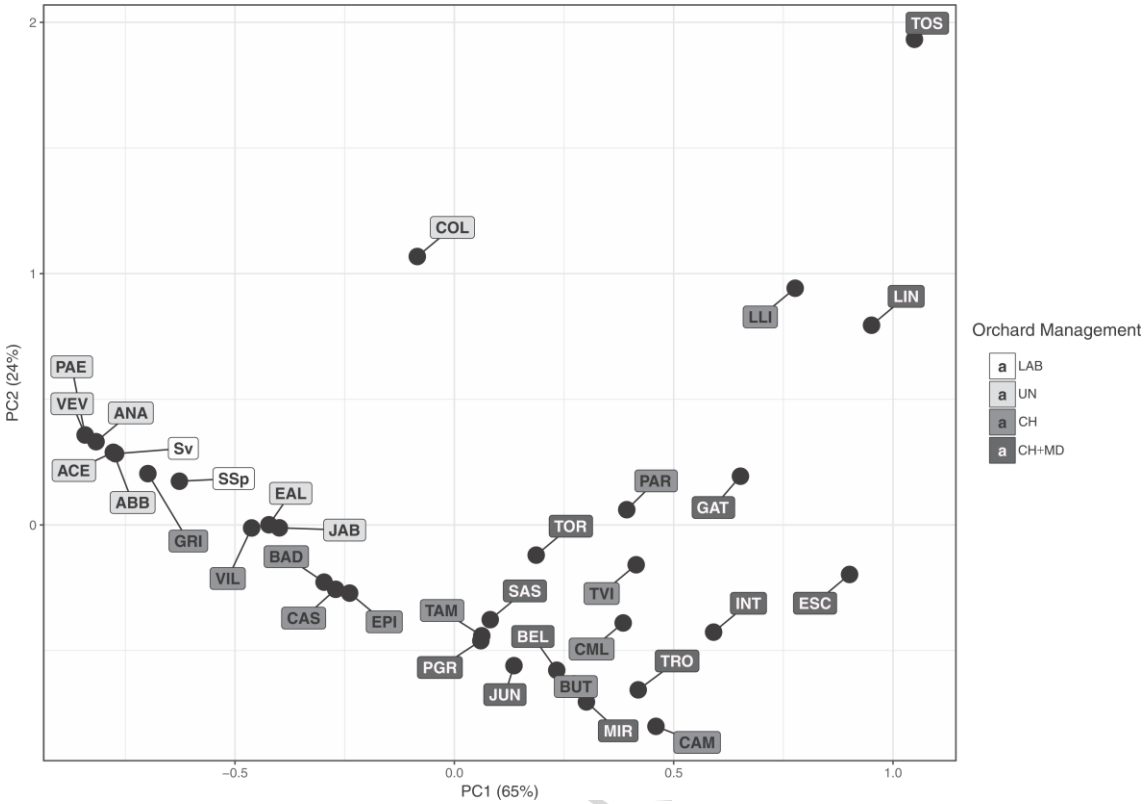


Figure 5

Table 1. Codling moth (*Cydia pomonella* (L.)) laboratory strains and Spanish field population location and management practices in the orchards.

Spanish region	Location	Population code	Management practices	Management code	Latitude - Longitude	Collection year
-	IRTA-Lleida, Spain	SSp	Laboratory	LAB	-	-
-	INRA-Avignon, France	Sv	Laboratory	LAB	-	-
Asturias	Villaviciosa, Gijón	ABB	Organic	UN	43º 31' 48.03" N - 5º 27' 05.18" W	2012
Asturias	Villaviciosa, Gijón	ACE	Organic	UN	43º 28' 33.24" N - 5º 26' 29.49" W	2012
Asturias	Nava, Gijón	ANA	Organic	UN	43º 02' 56.88" N - 5º 29' 32.69" W	2012
Asturias	Villaviciosa, Gijón	PAE	Organic	UN	43º 28' 28.51" N - 5º 26' 37.74" W	2010
Asturias	Villaviciosa, Gijón	VEV	Organic	UN	43º 28' 46.23" N - 5º 26' 31.25" W	2010
Extremadura	Badajoz, La Garrobilla	BAD	Chemically treated	CH	38º 54' 34.47" N - 6º 30' 41.35" W	2010
Aragon	Zaragoza, Figueruelas	CAS	Chemically treated	CH	41º 45' 46.85" N - 1º 11' 46.52" W	2010
Aragon	Zaragoza, Epila	EPI	Experimental treated	CH	41º 36' 34.96" N - 1º 13' 27.16" W	2010
Aragon	Zaragoza, Figueruelas	GRI	Chemically treated	CH	41º 45' 06.34" N - 1º 11' 48.99" W	2010
Aragon	Huesca, Tamarite	TAM	Chemically treated	CH	41º 51' 35.40" N - 0º 25' 41.85" W	2010
Aragon	Zaragoza, Villalengua	VIL	Chemically treated	CH	41º 25' 42.95" N - 1º 50' 05.13" W	2011
Catalonia	Lleida, Coll	COL	Organic	UN	41º 37' 41.43" N - 0º 32' 07.85" E	2010
Catalonia	Lleida, Almacelles	EAL	Organic	UN	41º 42' 11.86" N - 0º 29' 10.88" E	2010
Catalonia	Lleida, Juneda	JAB	Abandoned	UN	41º 32' 59.49" N - 0º 48' 38.59" E	2010
Catalonia	Lleida, Menàrguens	CAM	Chemically treated	CH	41º 43' 30.56" N - 0º 44' 48.19" E	2010
Catalonia	Lleida, Torre Villalba	TVI	Chemically treated	CH	41º 38' 19.75" N - 0º 34' 26.14" E	2010
Catalonia	Lleida, Termens	PAR	Chemically treated	CH	41º 42' 25.72" N - 0º 46' 16.04" E	2010
Catalonia	Lleida, Butsenit	BUT	Chemically treated	CH	41º 34' 37.11" N - 0º 34' 11.68" E	2010
Catalonia	Llivia, Lleida	LLI	Chemically treated	CH	41º 38' 41.46" N - 0º 38' 24.96 E	2010

Catalonia	Lleida, Menàrguens	CML	Chemically treated	CH	41º 43' 20.65" N - 0º 45' 31.14" E	2010
Catalonia	Lleida, Menàrguens	TRO	Mating Disruption + CH	MD+CH	41º 43' 20.61" N - 0º 44' 51.95" E	2010
Catalonia	Lleida, Termens	TOS	Mating Disruption + CH	MD+CH	41º 42' 38.83" N - 0º 47' 22.07" W	2010
Catalonia	Lleida, Linyola	PGR	Mating Disruption + CH	MD+CH	41º 41' 14.05" N - 0º 51' 52.10" W	2010
Catalonia	Lleida, Palau A.	GAT	Mating Disruption + CH	MD+CH	41º 39' 37.23" N - 0º 51' 25.92" E	2010
Catalonia	Llaida, Alpicat	INT	Mating Disruption + CH	MD+CH	41º 40' 14.75" N - 0º 32' 37.36" E	2010
Catalonia	Lleida, Tornabous	TOR	Mating Disruption + CH	MD+CH	41º 42' 24.49" N - 1º 03' 42.36" W	2010
Catalonia	Lleida, Palau A.	ESC	Mating Disruption + CH	MD+CH	41º 40' 6.30" N - 0º 51' 52.80" E	2010
Catalonia	Lleida, Bellvis	BEL	Mating Disruption + CH	MD+CH	41º 42' 30.66" N - 0º 50' 02.24" E	2010
Catalonia	Lleida, Linyola	LIN	Mating Disruption + CH	MD+CH	41º 40' 43.00" N - 0º 53' 19.76" E	2010
Catalonia	Lleida, Juneda	JUN	Mating Disruption + CH	MD+CH	41º 34' 56.86" N - 0º 52' 22.60 " E	2010
Catalonia	Lleida, Miralcamp	MIR	Mating Disruption + CH	MD+CH	41º 36' 48.21" N - 0º 52' 53.48" E	2010
Catalonia	Girona, St Andreu S.	SAS	Mating Disruption + CH	MD+CH	41º 52' 42.95" N - 2º 50' 5.13" W	2010

Table 2. Mean activity of PSMO (pg of 7-hydroxycoumarin insect^{-1} min^{-1}), resistance ratio (R/S) and frequency of resistant moths (R-PSMO), of 32 Spanish field populations and one susceptible laboratory strain (Sv) compared with the susceptible laboratory strain (SSp) of codling moth (LAB: laboratory strains, UN: non-chemically treated orchards, CH: chemically treated orchards, MD+CH: mating disruption plus chemically treated orchards).

Spanish region	Population code	Management code	n	Females (%)	PSMO activity Mean ± SE	Activity range	R-PSMO (%) [a]	R/S
-	SSp	LAB	34	49.0	9.1 ± 1.9	0 - 38.8	10.0	1.0
-	Sv	LAB	37	45.9	13.6 ± 2.0	0 - 38.8	8.1 ns	1.5
Asturias	ABB	UN	40	-	10.8 ± 2.1	0 - 52.3	7.5 ns	1.2
Asturias	ACE	UN	40	59.1	14.1 ± 2.5	0 - 63.8	7.5 ns	1.6
Asturias	ANA	UN	34	43.5	12.9 ± 2.1	0 - 46.7	2.9 * (<SSp)	1.4
Asturias	PAE	UN	21	42.9	8.8 ± 2.0	0 - 31.6	0.0 * (<SSp)	1.0
Asturias	VEV	UN	36	28.1	6.2 ± 1.2	0 - 30.8	0.0 * (<SSp)	0.7
Extremadura	BAD	CH	11	45.5	91.7 ± 24.7	6.8 - 279.7	63.6 ***	10.1
Aragon	CAS	CH	12	41.7	141.7 ± 39.6	7.7 - 414.3	66.7 ***	15.6
Aragon	EPI	CH	28	46.4	73.4 ± 10.2	0.7 - 179.0	64.3 ***	8.1
Aragon	GRI	CH	6	100.0	35.3 ± 11.6	5.7 - 78.3	16.7 n.s.	3.9
Aragon	TAM	CH	40	40.0	61.7 ± 10.2	0.8 - 294.2	50.0 ***	6.8
Aragon	VIL	CH	28	-	48.2 ± 13.9	0 - 307.8	32.1 **	5.3
Catalonia	COL	UN	38	13.2	21.1 ± 3.2	0 - 82.6	18.4 n.s.	2.3
Catalonia	EAL	UN	40	2.5	24.5 ± 3.9	0 - 103.7	19.0 n.s.	2.7
Catalonia	JAB	UN	40	2.5	22.4 ± 5.2	0 - 116.3	17.5 n.s.	2.5
Catalonia	CAM	CH	40	0.0	135.6 ± 21.1	0 - 507.4	75.0 ***	14.9
Catalonia	TVI	CH	37	5.4	137.2 ± 22.8	0 - 522.7	70.3 ***	15.1

Frequency of resistant individual was compared with the susceptible strain SSp using a χ^2 test: n.s. denotes no significant differences; P < 0.01**; P < 0.001***.

[a] <SSp denotes frequency of resistant individual lower than susceptible strain SSp.

Table 2 continuation. Mean activity of PSMO (pg of 7-hydroxycoumarin insect^{-1} min^{-1}), resistance ratio (R/S) and frequency of resistant moths (R-PSMO), of 32 Spanish field populations and one susceptible laboratory strain (Sv) compared with the susceptible laboratory strain (SSp) of codling moth (LAB: laboratory strains, UN: non-chemically treated orchards, CH: chemically treated orchards, MD+CH: mating disruption plus chemically treated orchards).

Spanish region	Population code	Management code	n	Females (%)	PSMO activity Mean ± SE	Activity range	R-PSMO (%) [a]	R/S
Catalonia	PAR	CH	43	18.6	90.1 ± 14.7	0 - 365.6	60.5 ***	9.9
Catalonia	BUT	CH	41	7.3	122.6 ± 21.9	0 - 532.6	63.4 ***	13.5
Catalonia	LLI	CH	40	17.5	127.4 ± 23.6	0 - 744.2	62.5 ***	14.0
Catalonia	CML	CH	31	12.9	90.9 ± 14.5	0 - 311.98	74.2 ***	10.0
Catalonia	TRO	MD+CH	38	10.5	137.9 ± 23.3	1.26 - 788.9	84.2 ***	15.2
Catalonia	TOS	MD+CH	37	27.0	94.36 ± 15.3	2.0 - 362.8	67.6 ***	10.4
Catalonia	PGR	MD+CH	37	21.6	100.0 ± 23.2	0 - 691.1	56.8 ***	11.0
Catalonia	GAT	MD+CH	38	13.2	127.0 ± 17.3	5.8 - 488.4	84.2 ***	14.0
Catalonia	INT	MD+CH	36	22.2	166.8 ± 24.9	0.8 - 463.6	72.2 ***	18.3
Catalonia	TOR	MD+CH	37	48.6	87.5 ± 14.1	1.2 - 323.4	62.2 ***	9.6
Catalonia	ESC	MD+CH	40	0.0	171.8 ± 20.4	17.8 - 575.1	90.0 ***	18.9
Catalonia	BEL	MD+CH	40	7.5	102.3 ± 18.8	1.4 - 424.12	55.0 ***	11.2
Catalonia	LIN	MD+CH	40	17.5	108.9 ± 18.9	1.0 - 529.65	70.0 ***	12.0
Catalonia	JUN	MD+CH	42	11.9	102.3 ± 14.6	0 - 435.0	71.4 ***	11.2
Catalonia	MIR	MD+CH	40	2.5	121.4 ± 16.2	4.8 - 491.95	80.0 ***	13.3
Catalonia	SAS	MD+CH	32	-	34.2 ± 8.7	0 - 167.4	25.0 ***	3.8

Frequency of resistant individual was compared with the susceptible strain using a χ^2 test: n.s denotes no significant differences; P < 0.01**; P < 0.001***.

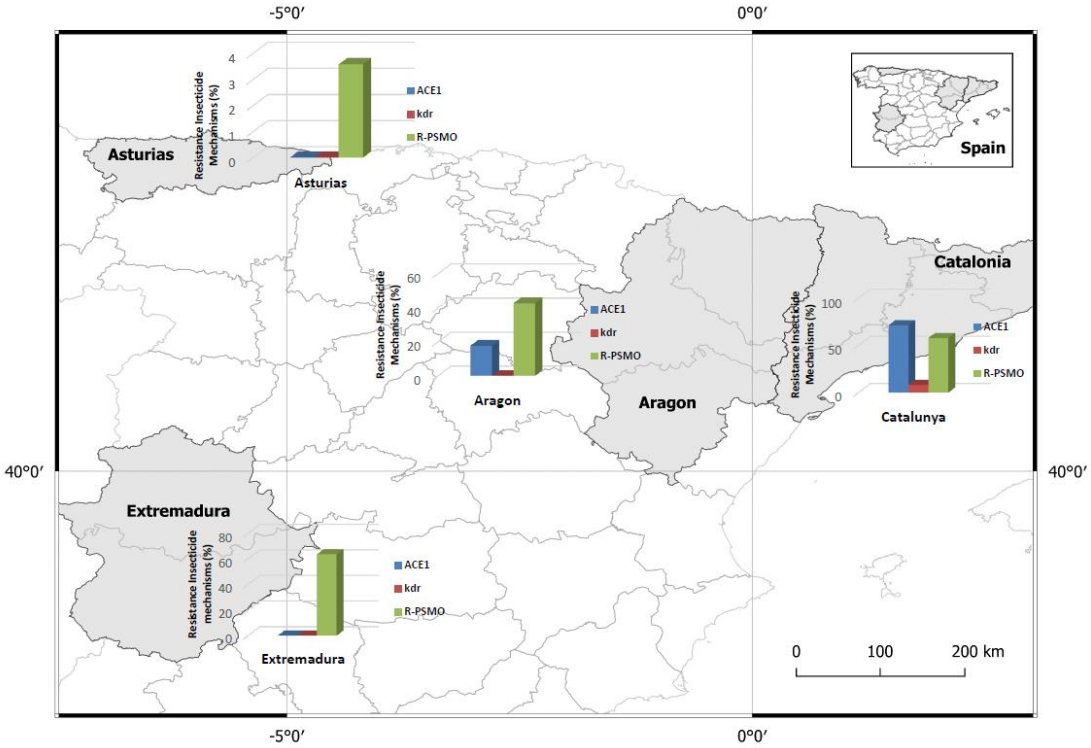
[a] <SSp denotes frequency of resistant individual lower than susceptible strain SSp.

Table 3. Genotype frequency (%) for *kdr* and *AChE* mutations of codling moth adults from two susceptible laboratory strains and 32 Spanish field-collected populations (LAB: laboratory strains, UN: non-chemically treated orchards, CH: chemically treated orchards, MD+CH: mating disruption plus chemical treated orchards).

Spanish region	Population code	Management code	n	*AChE*			*kdr*		
				RR	RS	SS	rr	rs	ss
-	SSp	LAB	34	0.0	0.0	100.0	0.0	0.0	100.0
-	Sv	LAB	37	0.0	0.0	100.0	0.0	0.0	100.0
Asturias	ABB	UN	40	0.0	0.0	100.0	0.0	0.0	100.0
Asturias	ACE	UN	40	0.0	0.0	100.0	0.0	0.0	100.0
Asturias	ANA	UN	34	0.0	0.0	100.0	0.0	0.0	100.0
Asturias	PAE	UN	21	0.0	0.0	100.0	0.0	0.0	100.0
Asturias	VEV	UN	36	0.0	0.0	100.0	0.0	0.0	100.0
Extremadura	BAD	CH	11	0.0	0.0	100.0	0.0	0.0	100.0
Aragon	CAS	CH	12	0.0	0.0	100.0	0.0	0.0	100.0
Aragon	EPI	CH	28	3.6	3.6	92.9	0.0	0.0	100.0
Aragon	GRI	CH	6	0.0	0.0	100.0	0.0	0.0	100.0
Aragon	TAM	CH	40	40.0	25.0	35.0	0.0	0.0	100.0
Aragon	VIL	CH	28	7.1	7.1	85.7	0.0	0.0	100.0
Catalonia	COL	UN	38	13.2	21.01	65.8	23.7	15.8	60.55
Catalonia	EAL	UN	40	10.0	25.0	65.0	0.0	20.0	80.0
Catalonia	JAB	UN	40	0.0	40.0	60.0	0.0	20.0	80.0
Catalonia	CAM	CH	40	2.5	87.5	10.0	0.0	0.0	100.0
Catalonia	TVI	CH	37	2.7	64.9	32.4	10.8	13.5	75.7
Catalonia	PAR	CH	43	0.0	69.8	30.2	14.0	14.0	72.1
Catalonia	BUT	CH	41	0.0	78.0	22.0	0.0	9.8	90.2
Catalonia	LLI	CH	40	2.5	75.0	22.5	35.0	17.5	47.5

Table 3 continuation. Genotype frequency (%) for *kdr* and *AChE* mutations of codling moth adults from two susceptible laboratory strains and 32 Spanish field-collected populations (LAB: laboratory strains, UN: non-chemically treated orchards, CH: chemically treated orchards, MD+CH: mating disruption plus chemical treated orchards).

Spanish region	Population code	Management code	n	*AChE*			*kdr*		
				RR	RS	SS	rr	rs	ss
Catalonia	CML	CH	31	0.0	67.7	32.3	6.5	6.5	87.1
Catalonia	TRO	MD+CH	38	68.4	28.9	2.6	2.6	7.9	89.5
Catalonia	TOS	MD+CH	37	18.9	45.9	35.1	56.8	13.5	29.7
Catalonia	PGR	MD+CH	37	2.7	54.1	43.2	0.0	75.7	24.3
Catalonia	GAT	MD+CH	38	47.4	15.8	36.8	21.1	39.5	39.5
Catalonia	INT	MD+CH	36	11.1	83.3	5.6	8.3	19.4	72.2
Catalonia	TOR	MD+CH	37	51.4	0.0	48.6	8.1	29.7	62.2
Catalonia	ESC	MD+CH	40	5.0	92.5	2.5	17.5	20.0	62.5
Catalonia	BEL	MD+CH	40	25.0	57.5	17.5	0.0	2.5	97.5
Catalonia	LIN	MD+CH	40	60.0	32.5	7.5	35.0	30.0	35.0
Catalonia	JUN	MD+CH	42	0.0	76.2	23.8	0.0	2.4	97.6
Catalonia	MIR	MD+CH	40	5.0	57.5	37.5	0.0	0.0	100.0
Catalonia	SAS	MD+CH	32	87.5	9.4	3.1	0.0	0.0	100.0



Graphical abstract

Highlights

- High frequency of AChE mutation was present in the Ebro Valley populations.
- *Kdr* mutation was also extended in Catalonia populations.
- High PSMO activity was widely extended in all insecticide sprayed orchards.
- Mutations and the enzymatic resistance mechanism (PSMO) appeared combined.
- Codling moth adult's dispersion plays an important role in the extent of resistance.